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The Effects of Exogenous Auxins on Endogenous Indole-3-Acetic Acid Metabolism[1]

The Implications for Carrot Somatic Embryogenesis

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The effect of auxin application on auxin metabolism was investigated in excised hypocotyl cultures of carrot (*Daucus carota*). Concentrations of both free and conjugated indole-3-acetic acid (IAA), [$^2H_4$]IAA, 2,4-dichlorophenoxyacetic acid, and naphthaleneacetic acid (NAA) were measured by mass spectroscopy using stable-isotope-labeled internal standards. [$^{13}C_1$]NAA was synthesized for this purpose, thus extending the range of auxins that can be assayed by stable-isotope techniques. 2,4-Dichlorophenoxyacetic acid promoted callus proliferation of the excised hypocotyls, accumulated as the free form in large quantities, and had minor effects on endogenous IAA concentrations. NAA promoted callus proliferation and the resulting callus became organogenic, producing both roots and shoots. NAA was found mostly in the conjugated form and had minor effects on endogenous IAA concentrations. [$^2H_4$]IAA had no visible effect on the growth pattern of cultured hypocotyls, possibly because it was rapidly metabolized to form inactive conjugates or possibly because it mediated a decrease in endogenous IAA concentrations by an apparent feedback mechanism. The presence of exogenous auxins did not affect tryptophan labeling of either the endogenous tryptophan or IAA pools. This suggested that exogenous auxins did not alter the IAA biosynthetic pathway, but that synthetic auxins did appear to be necessary to induce callus proliferation, which was essential for excised hypocotyls to gain the competence to form somatic embryos.

Since the 1940s (Thimann, 1974), exogenous auxins have been used frequently as experimental tools for studying their effects in developing structures (Schiavone and Cooke, 1987); for screening for mutants impaired in the uptake, metabolism, and physiology of endogenous auxins (Timpte et al., 1994); and even to find potential IAA receptors (Jones and Venis, 1989; LoSchiavo et al., 1991). One important use for the application of exogenous auxin is as a growth regulator for maintaining plant-cell- and tissue-culture systems. In such culture systems, added auxin is generally associated with the promotion of growth, callus proliferation, and the induction of rooting. Despite the wide use of exogenous auxins in cell culture, little is known about how they affect the metabolism of endogenous auxins.

2,4-D is commonly used to initiate tissue cultures of carrot (*Daucus carota*) (Ammirato, 1985) and other species (Dunstan et al., 1995). The presence of 2,4-D in the growth medium often results in undifferentiated callus proliferation. Upon the removal of 2,4-D from the growth medium of carrot cultures, undifferentiated cell clusters may commence an organized growth pattern and develop into somatic embryos. In contrast, NAA has been demonstrated to be an effective auxin for both the initiation and maturation of somatic embryos in carrot (Ammirato, 1985). In certain species of the Gramineae, 2,4-D has also been used to initiate the process of embryogenesis (Vasil, 1985), but the 2,4-D concentration must be lowered, not eliminated, in order to promote the development of somatic embryos. Thus, a basic requirement for a synthetic auxin treatment seems to be an underlying feature of somatic embryogenesis (Ammirato, 1985) in all cell cultures examined to date.

Not only is the exogenous auxin important in the initiation of somatic embryogenesis, but changes in endogenous auxin concentrations may also be critical for the later stages of embryogenesis as well (Cooke et al., 1993). It has been shown that embryogenic carrot cells grown in the presence of 2,4-D contain large amounts of IAA (Michalczuk et al., 1992b; Sasaki et al., 1994). The transfer of embryogenic cell clusters to medium lacking 2,4-D, which initiated the process of somatic embryo formation, was shown to be coincident with a large decrease in endogenous IAA (Michalczuk et al., 1992b). A high IAA concentration may be required to induce the competence of cells to undergo somatic embryogenesis, and the subsequent decrease of IAA may be required for the development of the somatic embryos that follows. What is not clear, however, is how the relationship between exogenous auxins and endoge-

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Abbreviations: MS, Murashige and Skoog; m/z, mass to charge ratio; SPE, solid phase extraction.

nous auxins might function to control the process of somatic embryogenesis.

In this paper, the effect of exogenous auxins on endogenous IAA metabolism was examined by culturing excised carrot hypocotyls on media containing either no auxins, 2,4-D, NAA, or [$^2H_4$]IAA. Excised hypocotyls were used rather than cultured cells because these experiments required a plant material with no prior exposure to synthetic auxins. The concentrations of free and conjugated IAA were then determined for each treatment at both 1- and 4-week incubation periods. Because the exogenous auxins could act either indirectly by affecting endogenous IAA metabolism (Fujimura and Komamine, 1979; Moloney et al., 1983; Michalczuk et al., 1992b, 1992c) for more directly as auxin analogs, it was important to measure the in situ concentrations of the exogenous auxins as well. The use of deuterium-labeled IAA as the exogenous auxin allowed us to simultaneously monitor the concentrations of both the unlabeled native IAA and the labeled exogenous IAA in a manner similar to the procedure described by Meuwly and Pilet (1991). The concentrations of all the auxins plus their conjugates were quantified on the basis of stable-isotope dilution of ^{13}C-labeled internal standards. No ^{13}C-labeled internal standard was commercially available for NAA, so [$^{13}C_1$]NAA was synthesized for use in these measurements.

The embryogenic potential of the callus derived from each auxin treatment was also evaluated by transferring the callus to an auxin-free medium and observing subsequent morphological events. Since this process is known to produce embryos from 2,4-D-treated hypocotyls, the other treatments were evaluated with respect to the 2,4-D effect. The experiments were designed to test whether the prerequisite high concentration of auxins, which seemed to mark the onset of embryogenesis, could be satisfied by only one or several exogenous auxins.

In a previous report, we found that a change in the pathway for IAA biosynthesis occurred during the process of somatic embryogenesis (Michalczuk et al., 1992b). When carrot suspension cultures were proliferating in the presence of 2,4-D, endogenous IAA was derived primarily from Trp, but a non-Trp pathway became the dominant source of the endogenous IAA in developing embryos. It was not clear whether the removal of the 2,4-D, the decrease in endogenous IAA concentration, or the formation of somatic embryos was most closely integrated with the shift in the biosynthetic pathway. To assess the possible involvement of synthetic auxins in this shift, the ability of [$^2H_5$]L-Trp to label both the endogenous Trp pool and the endogenous IAA pool was measured in both the presence and absence of exogenous auxins.

The above experimental approaches allowed us to dissect various aspects of auxin metabolism associated with somatic embryogenesis to determine which were integral to the process and which were the result of it. In doing so, we hoped to further elucidate the role of auxin in somatic embryogenesis, as well as to address some basic assumptions implicit in the use of exogenous auxins.

MATERIALS AND METHODS

Hypocotyl Experiment

Carrot seeds (*Daucus carota* L. cv Danvers, Meyers Seed,[2] Baltimore, MD) were sterilized in 5% NaOCl (Clorox) containing 1% Triton X-100 (Sigma) for 25 min, rinsed, and germinated for 7 d on deionized water solidified with 1% agar. Hypocotyl segments (1–2 cm) were excised under sterile conditions and transferred to fresh plates containing MS basal salt medium (Murashige and Skoog, 1962) with 3% Suc and supplemented either with no auxins or with 2,4-D, [$^2H_4$]IAA, or NAA (at a concentration of 10 mM). In parallel, hypocotyls were also cultured under the same conditions but with the addition of 250 mM [$^2H_5$]L-Trp to the medium. The excised hypocotyls were then analyzed for endogenous concentrations of Trp, endogenous IAA, and exogenous auxins after 1 week, and the same auxins after 4 weeks. After 4 weeks, small pieces of callus from each of the treatments were transferred to MS medium lacking growth-regulator supplements to assess the embryogenic potential of the callus. All of the cultures were incubated in the dark at 25°C, but all manipulations were performed in ambient light. Photomicrographs were taken of the hypocotyls after the 4-week treatment and 4 weeks after transfer of the callus to the MS medium with no hormone supplement.

Auxin Determination

IAA, 2,4-D, and NAA were extracted from the hypocotyls in 65% isopropanol/0.2 M imidazole buffer, pH 7.0, containing the appropriate ^{13}C-labeled internal standard(s) (Cohen et al., 1986) at a concentration of 200 ng/g fresh weight as described by Chen et al. (1988). The free auxins were purified on an amino SPE column (PrepSep, Fisher) followed by HPLC on a ODS 30 column (Ultracarb 5, Phenomenex, Torrance, CA) (50 × 4.6 mm) with 25% methanol/water containing 1% acetic acid as a running solvent and a flow rate of 1 mL/min. Under these conditions, IAA had an elution time of 10 to 12 min.

For each of the treatments, the free IAA was measured as well as the free + ester and total IAA (free + ester + amide-conjugated IAA). The concentrations of the bound forms of IAA (ester and amide conjugates) were obtained to detect possible perturbations in this aspect of IAA metabolism and metabolic regulation. The free + ester-conjugated fraction was obtained by analysis of IAA following a mild hydrolysis (25°C, 1 N NaOH, 1 h), and the total auxin fraction (free + ester conjugates + amide conjugates) was obtained by analysis of IAA following a harsh hydrolysis (100°C, 7 N NaOH, 3 h), as previously described (Cohen et al., 1986). Both the free + ester and the total fractions were titrated to pH 2.5 with 2 N HCl, desalted with an SPE column (Fisher), and purified by HPLC as described above for the free auxins.

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

2,4-D and NAA were extracted and prepared simultaneously with IAA (as described above). The sample was injected into the HPLC column, and the 2,4-D or the NAA were collected at either 5 or 8 min, respectively, after a change in elution solvent to 100% methanol at 20 min.

Endogenous IAA concentrations were determined using [$^{13}C_6$]IAA as an internal standard as described by Chen et al. (1988). After methylation, GC-MS-selected ion monitoring analysis of the sample was performed on a GC-MS system (5890/5971A, Hewlett-Packard) equipped with a fused silica capillary column (15 m × 0.237 mm, DB-1701, J&W, Folsom, CA). The ions monitored for the determination of IAA concentration were m/z 130 and 136 (quinolinium ions from the unlabeled and labeled methylated IAAs, respectively), and 189 and 195 (molecular ion and $M^+ + 6$). Similarly, 2,4-D concentrations were determined using a [$^{13}C_6$]2,4-D internal standard (Cambridge Isotopes, Andover, MA) by monitoring the ions m/z 234 and 240 (molecular ion and $M^+ + 6$), and NAA concentrations were determined by using the custom-synthesized [$^{13}C_1$]NAA (described below) as the internal standard by monitoring the ions m/z 200 and 201 (molecular ion and $M^+ + 1$). All of the values for auxin concentrations are reported as the mean ± SE for 3 to 10 replicate experiments.

[$^{13}C_1$]NAA Synthesis

[$^{13}C_1$]NAA was synthesized by a modification of the reaction described by Cambron (1939) in two steps using α-chloromethylnaphthalene and K[^{13}C]N as starting compounds. In a 2-mL flask equipped with a reflux condenser, a mixture of 223 mg of α-chloromethylnaphthalene (Aldrich) and a solution of 110 mg K[^{13}C]N (99 atom %, Cambridge Isotopes) in 0.5 mL of ethyl alcohol and 0.2 mL of water was refluxed for 1 h. The alcohol was removed using a stream of N_2 with a 50°C sand bath, giving a light yellow oil as the product. The product, α-naphthaleneacetonitrile, was checked on silica-gel TLC plates (Merck, Darmstadt, Germany) with a solvent of 5 isopropanol:30 hexane by comparing its R_F to that of an indoleacetonitrile standard that has been noted to have a similar R_F. The hydrolysis of α-naphthlaleneacetonitrile to α-NAA was conducted by refluxing the light yellow oil with a mixture of 96% reagent-grade sulfuric acid (0.35 mL), glacial acetic acid (0.35 mL), and water (0.35 mL) at 120 to 125°C for 45 min.

The acidic mixture was partitioned with diethylether (3 × 10 mL), the ether was removed in vacuum, and the product was dissolved in distilled water. This solution was neutralized to pH 7.0 and extracted again with ether. The ether phase was again evaporated and the product was redissolved in water. The solution was acidified to pH 2.0 and partitioned again with ether; the product was in the ether phase. The solvent was removed, and the remaining product was recrystallized from 50% ethanol, giving small, white crystals of α-NAA. A sample of the product was methylated using diazomethane (Cohen, 1984), and its identity was confirmed by GC-MS. Figure 1 shows a mass spectrum (A) and chromatogram (B) of the methyl-[$^{13}C_1$]NAA internal standard. The ion of mass 201 represents the molecular ion of the internal standard, which is 1 mass unit heavier than the molecular ion of the unlabeled methyl-NAA. The NAA internal standard was always used at concentrations greater than that expected in the plant material to avoid error resulting from naturally occurring ^{13}C abundance, as described by Sutter and Cohen (1992).

Trp Analysis

Trp was extracted from the hypocotyls after 1 week of treatment in 65% isopropanol/0.2 M imidazole buffer, pH 7.0, as was done for the auxin analysis. Extracts were concentrated on a rotary evaporator under vacuum to approximately one-third of the initial volume and applied to an 8-mL bed volume column (50W-X2, 200–400 mesh, H^+ form, Dowex, Sigma). The column was washed with 3 bed volumes of distilled water and eluted with 20 mL of 2 N NH_4OH. The Trp sample was evaporated to dryness and derivatized with methanolic acetic anhydride (Michalczuk et al., 1992a) to form the methyl ester of *N*-acetyl Trp, and the product was purified by HPLC. GC-MS analysis was performed as described above, except that the ramp rate was 30°C/min. Under these conditions the retention time for the methyl ester of *N*-acetyl Trp was 6.08 min. The ions that were monitored included m/z 130 and 135 (quino-

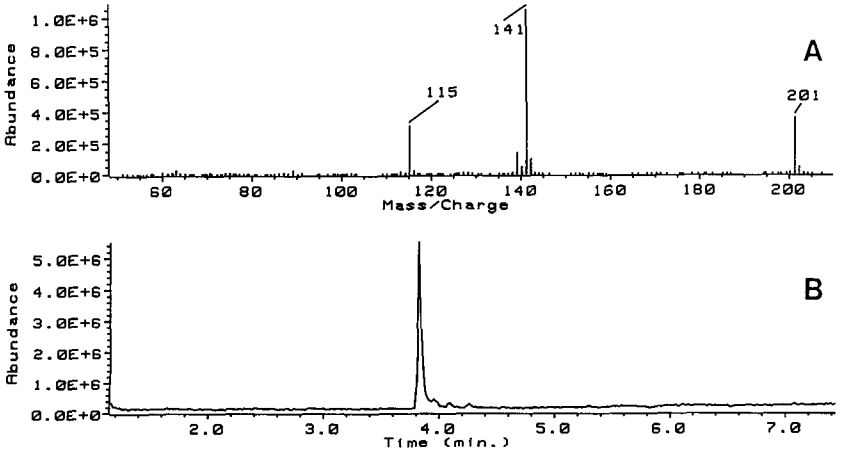

Figure 1. Mass spectrum (A) and total ion chromatogram (B) of [$^{13}C_1$]NAA synthesized as an internal standard for the quantitation of NAA extracted from carrot cultures.

linium ion and $M^+ + 5$) and m/z 260 and 265 (molecular ion and $M^+ + 5$).

RESULTS

IAA Measurements

Endogenous IAA concentrations were determined in carrot hypocotyls cultured on MS medium containing 10 mM 2,4-D, NAA, or [$^2H_4$]IAA, as well as control medium lacking exogenous auxin supplement (Fig. 2). The free, free + ester-conjugated IAA, and total IAA concentrations were determined. The concentration of the ester-conjugated IAA can be obtained by subtracting the amount of free IAA from the free + ester-conjugated IAA concentration. The concentration of the amide-conjugated IAA can be obtained by subtracting the free + ester-conjugated IAA from the total IAA concentration. Measurements were made at 1- and 4-week time periods to observe both the short- and long-term responses to the exogenous auxins.

During the 1st week of incubation, the exogenous auxins had no major effect on the level of endogenous IAA (Fig. 2).

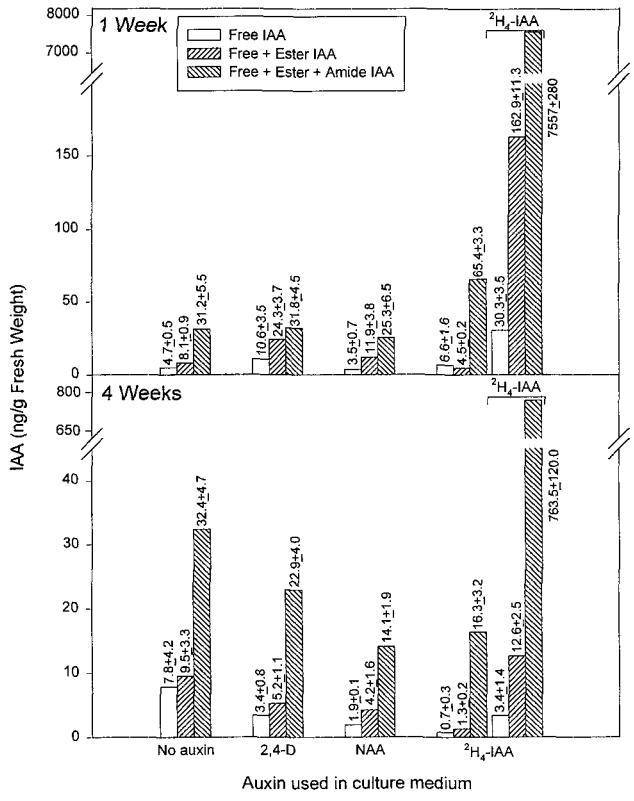

Figure 2. The free, free + ester-conjugated, and free + ester + amide IAA concentrations (ng/g fresh weight) following 1- and 4-week treatments of hypocotyls cultured with either no auxin, 2,4-D, NAA, or [$^2H_4$]IAA are shown. The ester-conjugate concentration is the difference between the free and free + ester-conjugate IAA concentrations. The amide-conjugate concentration is the difference between the free + ester-conjugate and total IAA concentrations. The endogenous IAA and the [$^2H_4$]IAA were quantified separately in hypocotyls treated with[$^2H_4$]IAA. Values are derived from 3 to 10 replicate experiments ± SE.

2,4-D- and NAA-treated hypocotyls contained slightly increased concentrations of ester-bound IAA and slightly lower concentrations of amide-bound IAA when compared with the no-auxin control. Hypocotyls grown on medium containing 2,4-D also had a slight increase in the concentration of free IAA, whereas [$^2H_4$]IAA-treated hypocotyls had elevated concentrations of all forms of IAA. It was not clear, however, if the observed changes in IAA concentrations were biologically significant after the 1-week treatment period, since the hypocotyls from all treatments consistently appeared slightly swollen in comparison with freshly excised hypocotyls.

In addition to determining the levels of endogenous IAA, [$^2H_4$]IAA levels were measured for the [$^2H_4$]IAA-treated hypocotyls to determine how the cultures responded to an imposed increase in the concentration of the free IAA (Fig. 2). The concentration of the free, ester-bound, and amide-bound [$^2H_4$]IAA were all much higher than the corresponding concentration of the endogenous forms of IAA in the 1-week cultures. The conjugated forms of [$^2H_4$]IAA were 250 times more abundant than free [$^2H_4$]IAA, suggesting that [$^2H_4$]IAA, and therefore IAA, induced its own conjugation, as was also observed by Venis (1972). This would explain the increase noted in the concentration of amide-conjugated, unlabeled IAA as well.

2,4-D treatment had a minor positive effect on the endogenous free and ester-conjugate IAA concentrations during the 1st week, which seemed to subsequently disappear by the 4th week (Fig. 2). NAA-treated hypocotyls showed a slight decrease in the free and total IAA pools. The endogenous free and ester-bound IAA concentrations were, in contrast, greatly reduced in the [$^2H_4$]IAA-treated cultures after 4 weeks, possibly resulting from a decrease in endogenous IAA biosynthesis. When the endogenous IAA and [$^2H_4$]IAA were considered together as the complete IAA pool, however, the free and ester-bound IAA concentrations were roughly comparable to the endogenous IAA concentrations found with the other treatments. The total IAA concentration for the [$^2H_4$]IAA treatment was reduced 10-fold during the 1st 1 to 4 weeks, but was still substantial at 4 weeks and consisted almost exclusively of amide-conjugated [$^2H_4$]IAA.

Exogenous Auxin Measurements

The concentrations of the exogenous auxins (2,4-D and NAA in addition to [$^2H_4$]IAA) in the hypocotyls were also measured after 1 and 4 weeks of culture (Table I). After the 1-week culture period, 2,4-D was present in high amounts as the free compound, which suggested that carrot hypocotyls lacked a constitutive mechanism for conjugating 2,4-D or that 2,4-D conjugates, if formed, were rapidly hydrolyzed. NAA and [$^2H_4$]IAA were found in lesser, but nevertheless substantial, amounts after a 1-week culture period. Unlike 2,4-D, NAA and [$^2H_4$]IAA were found predominantly in conjugated forms. Thus, NAA was either conjugated via the same pathway as IAA or the enzymes involved in its conjugation were more readily induced than those modifying 2,4-D. Nevertheless, the free NAA concentration (Table I) was more than 1 order of magnitude

Table I. *Exogenous auxin concentration in cultured carrot hypocotyls*

The free and total exogenous auxin concentrations (ng/g fresh weight) following 1- and 4-week treatments of hypocotyls cultured with either 2,4-D, NAA, or [$^2H_4$]IAA as determined by GC-MS-SIM are shown. All values are means of three to seven measurements ± SE.

Treatment Period	Exogenous Auxin Concentrations		
	Auxin treatment	Free auxin	Total
		ng/g fresh wt	
1 Week	2,4-D	23,247.4 ± 3,912.7	21,979.4 ± 2,526.3
	NAA	99.3 ± 9.7	1,776.7 ± 50.1
	[$^2H_4$]IAA	30.3 ± 3.5	7,557.0 ± 279.0
4 Weeks	2,4-D	20,003.8 ± 4,365.9	33,119.4 ± 3,835.0
	NAA	64.8 ± 17.5	1,637.0 ± 509.8
	[$^2H_4$]IAA	3.4 ± 1.4	763.5 ± 120.0

higher than the typical IAA concentration observed in untreated cultures (Fig. 2). After 1 week of treatment (Table I), the initial concentration of total [$^2H_4$]IAA was very high, whereas the concentration of the free [$^2H_4$]IAA was low, indicating an efficient conjugation mechanism.

After 4 weeks of culture, the concentration of free 2,4-D remained relatively constant, but over 35% of the total 2,4-D pool consisted of conjugated forms. The concentration of free NAA decreased slightly, whereas the total NAA pool remained constant. In contrast, the concentrations of both the free and bound forms of [$^2H_4$]IAA decreased dramatically. In summary, the free 2,4-D concentration remained exceptionally high over the 4-week culture period, the free and total NAA concentrations remained moderate, and the free [$^2H_4$]IAA concentration decreased to very low levels.

Morphological Observations

The developmental consequences of the auxin treatments are shown in the photomicrographs in Figure 3. As was previously mentioned, all 1-week treatments were noted by the slight swelling of the hypocotyls with no obvious differences among treatments (data not shown). Hypocotyls cultured for 4 weeks in the absence of any exogenous auxins produced small amounts of callus at both cut ends and roots at the basal end (Fig. 3A). When hypocotyls were cultured in the presence of 10 mM 2,4-D, callus started to proliferate at the cut ends and then throughout the entire hypocotyl (Fig. 3B). Hypocotyls cultured in the presence of 10 mM [$^2H_4$]IAA for 4 weeks appeared very much like those cultured in the absence of any auxin, with small amounts of callus at the cut ends and many roots proliferating after 4 weeks (Fig. 3C). The NAA treatment, however, induced larger amounts of callus growth than the 2,4-D treatment, and the resulting callus produced both roots and embryos at 4 weeks (Fig. 3D). Although microscopic observations confirmed the presence of embryos in these cul-

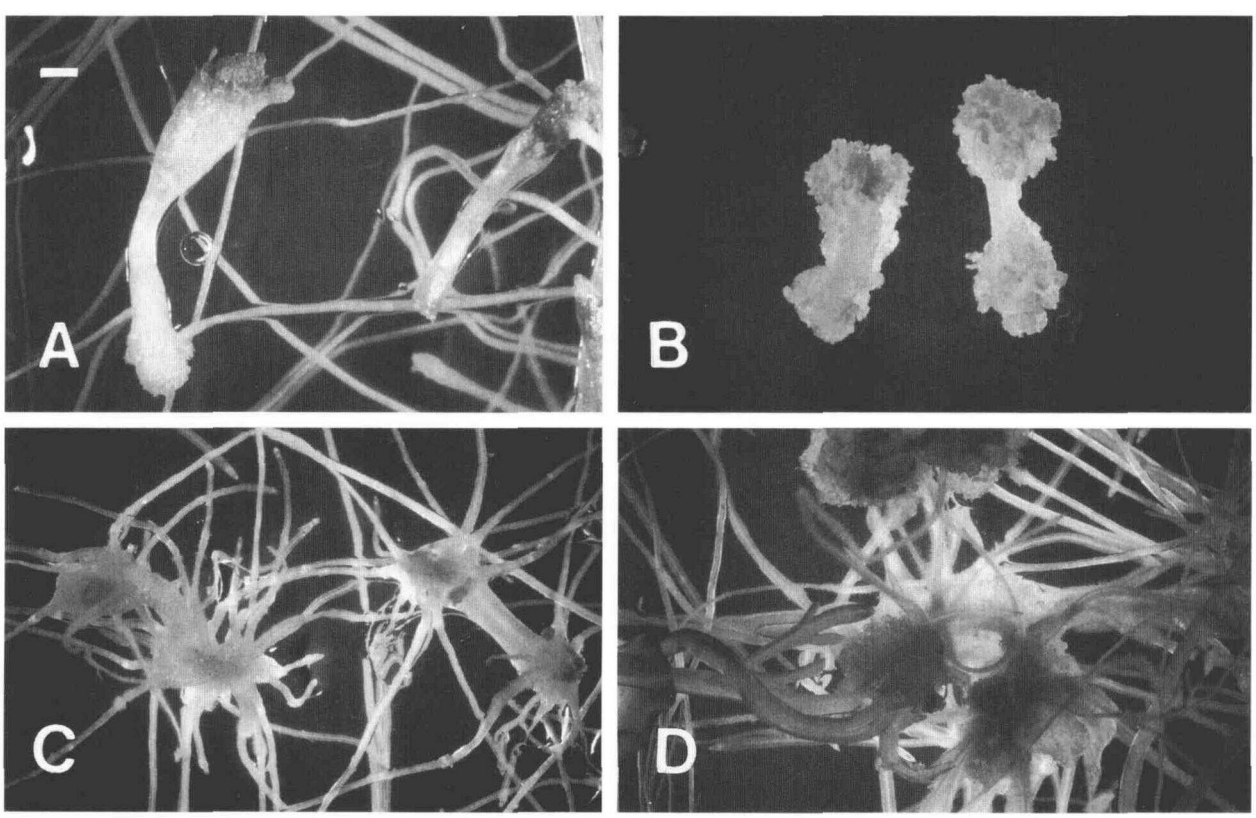

Figure 3. Photomicrographs of hypocotyls cultured in MS medium for 4 weeks with either no auxin (A) or with 2,4-D (B), NAA (C), or [$^2H_4$]IAA (D). The bar at the upper left in A = 1 mm.

tures, we cannot eliminate the possibility that some shoots also arose as independent organs.

To examine the embryogenic potential of the various cultures, small amounts of callus from each culture after 4 weeks of treatment were transferred to the medium with no added auxin. The photomicrographs of Figure 4 were taken after 4 weeks of secondary culture without auxin. It should be noted that it was difficult to obtain much, if any, callus exclusive of organized hypocotyl cells from cultures without auxin and from the [$^2H_4$]IAA-treated cultures. The cellular material transferred from the culture without exogenous auxin did not grow and appeared senescent after 4 weeks. The callus transfered from the 2,4-D-treated hypocotyls proliferated embryos (Fig. 4A). The cellular material from the [$^2H_4$]IAA-treated hypocotyls in general did not grow at all and most of the cellular material senesced after 4 weeks, as did the control cultures with no auxin treatment. However, in 1 of the 12 replicates, a single piece of callus produced two embryo-like structures. Callus transferred from NAA-treated hypocotyls continued to proliferate shoots and roots (Fig. 4B). Again, it was difficult to determine if true bipolar embryos with interconnected root and shoot poles were formed from the NAA-treated callus, since small amounts of callus proliferated in crowded masses of organized structures, making it difficult to distinguish individual structures. Nevertheless, the organogenic nature of NAA-derived callus could be equated with embryogenic material with respect to its ability to induce organized growth from undifferentiated callus.

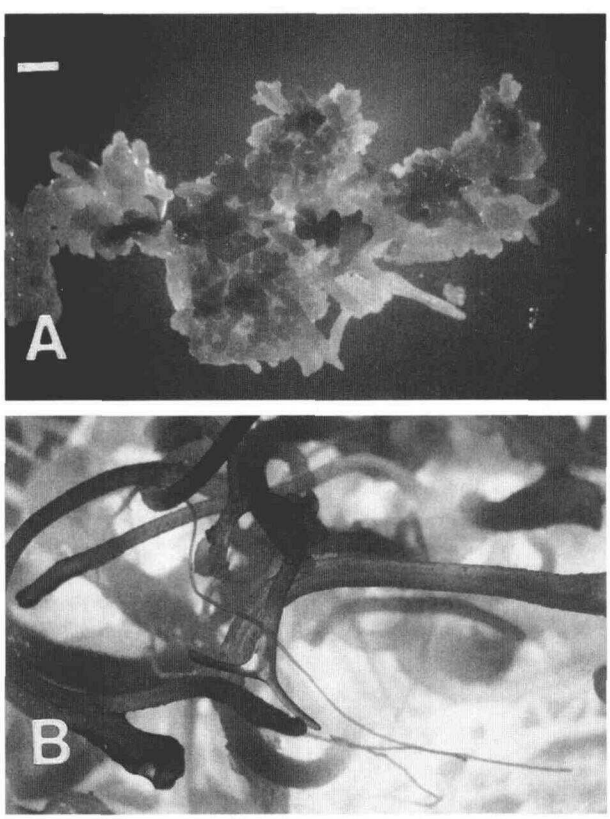

Figure 4. Photomicrographs of callus cultured for 4 weeks in MS medium with no auxin after a 4-week pretreatment with either 2,4-D (A) or NAA (B). The bar at the upper left in A = 1 mm.

Since NAA and 2,4-D are synthetic auxins that are metabolized differently by hypocotyl cultures (Table I) and elicit very different responses in those cultures (Fig. 3), it was worthwhile to evaluate the metabolic and developmental interactions between these two synthetic auxins. Therefore, a trial experiment was conducted in which hypocotyls were cultured on MS medium containing both 2,4-D and NAA (10 mM). After 4 weeks of culture, the concentrations of the free and conjugated endogenous IAA were within 10% of those found in the 2,4-D treatment (Fig. 2). The concentrations of free NAA (119 ng/g fresh weight) and conjugated NAA (3974 ng/g fresh weight) were essentially doubled relative to the experiment with NAA alone. The concentration of free 2,4-D in this combined auxin experiment at 4 weeks was also almost double the amount found with 2,4-D alone, 41,800 ng/g fresh weight. The callus proliferation of these cultures exactly matched the cultures grown in the presence of 2,4-D alone. Thus, these two auxins did not appear to have any qualitative effects on their respective metabolisms, but the callus formation induced by the 2,4-D treatment did dominate over the organized growth associated with the NAA treatment. Recent work, however, by Lou and Kako (1995) in cucumber showed that treatments with different combinations of auxins had dramatically different effects on the yield of somatic embryos, which suggests that each auxin has a distinct action.

Trp Labeling

To evaluate if auxin application had any effect on the biosynthesis of IAA from Trp, the ability of Trp to label both the Trp and IAA pools was measured for each of the 1-week auxin treatments. [$^2H_5$]L-Trp enriched the endogenous Trp pool from 47 to 48% in the absence of exogenous auxin and in the presence of 10 mM 2,4-D or NAA. Therefore, the presence of exogenous auxins did not affect the ability of exogenous Trp to label the endogenous Trp pool. [$^2H_5$]L-Trp labeled the endogenous IAA pools to a similar extent for each of the treatments as well. $^2H_5$ labeling of the endogenous IAA pool from exogenous Trp in untreated hypocotyls was 47.2 ± 0.7%, in 2,4-D-treated hypocotyls it was 43.5 ± 3.7%, and in NAA-treated hypocotyls it was 45.7 ± 1.7%. These percentages are based on the sum of the measured ion abundance at m/z 130 and 135 being 100%.

DISCUSSION

The presence of exogenous auxins in the culture medium of carrot hypocotyls had profound effects on the morphological appearance of the resulting cultures (Fig. 3). All of the exogenous growth regulators were present in planta in very different total concentrations, which would be attributable to differential rates of auxin degradation in the medium, and of uptake and catabolism following uptake. The observation that all of the exogenous auxins accumulated in large quantities suggests that auxin uptake into

plant material was an efficient process. Despite possible differences in auxin uptake, metabolism must be a significant factor affecting the concentrations of these auxins.

2,4-D

2,4-D treatment had only minor effects on the concentration of endogenous IAA after either 1 or 4 weeks of culture, suggesting that 2,4-D must have acted directly as the auxin in carrot hypocotyls and not by inducing changes in endogenous IAA concentration. The activity of the applied auxins must be viewed with consideration of their metabolism and persistence (Table I). Of the auxins tested, 2,4-D accumulated to the highest concentration. The concentration of 2,4-D was similar at both 1 and 4 weeks, but only after the 4-week incubation period were significant amounts of conjugated 2,4-D detected. The lack of 2,4-D conjugation with short-term incubation of carrot cells was previously noted by Sasaki et al. (1993), but they did not examine long-term metabolism.

The slow formation of conjugates of 2,4-D contrasts with the more rapid conjugation found with IAA and NAA, suggesting that the processes are very different. In soybean callus cultures, one of the few plant cells in which 2,4-D metabolism has been studied, the pool of free 2,4-D was metabolized to form 2,4-D conjugates with glutamic (Feung et al., 1971) and aspartic acids (Feung et al., 1972). Like conjugates of IAA (Bialek et al., 1983), 2,4-D conjugates could presumably be hydrolyzed back to the free form. The catabolism of 2,4-D into the predominant glycoside of 4-hydroxy-2,5-dichloroacetic acid and the less-abundant glycoside of 2,3-dichlorophenoxyacetic acid is one well-defined degradative pathway in soybean cultures (Feung et al., 1971, 1972; Hamilton et al., 1971). However, in general, 2,4-D is a relatively stable compound that has been shown to be very persistent in carrot cultures (Michalczuk et al., 1992b).

2,4-D is considered to be a "strong" auxin in culture (Ammirato, 1985) because its effects are so pronounced. The biological reason for this may result from the high concentrations of free 2,4-D, which are maintained in cultures over long periods of time (Michalczuk et al., 1992b). Uncertainty exists, however, since the effects of 2,4-D in tissue culture seem to require high concentrations of the growth regulator. If 2,4-D is removed from tissue culture growth medium, the growth effects of the 2,4-D are lost even though the plant materials contain significant quantities of 2,4-D (Michalczuk et al., 1992b). Exogenous free 2,4-D seems to be required in a large quantity for activity, often accumulates in planta in large quantities, and is not easily conjugated by carrot hypocotyls. These properties suggest that the metabolism and associated activity of 2,4-D in culture are very different from that of applied IAA and NAA.

NAA

NAA accumulated in much lower total amounts than 2,4-D, and the amount of free NAA was much less than the amount of conjugated NAA. Nevertheless, the concentration of free NAA was significantly higher than the concentration of free IAA in untreated cultures, and, therefore, the free NAA seemed directly responsible for the observed auxin-like changes in the growth of these cultures. NAA, like IAA, has been shown to induce its own conjugation when applied to plants, thereby increasing the capacity for the formation of NAA-aspartate (Südi, 1966; Johansson, 1971; Venis, 1972; Brenner and Tonkinson, 1974; Smulders et al., 1990). Cultured carrot hypocotyls conjugated NAA much more readily than they did 2,4-D, which in part accounted for the low free NAA content. NAA has been used in similar experimental systems to promote the growth of somatic embryos as well as the proliferation of shoots and has often been used in conjunction with cytokinins (Yuan et al., 1994).

NAA treatment, like 2,4-D treatment, did not have any profound effect on the metabolism of endogenous IAA, but hypocotyls showed a 50% decrease in the concentration of all forms of IAA after 4 weeks of treatment. In this respect, the NAA treatment appeared to be like the [$^2H_4$]IAA treatment and resulted in a possible decrease in the biosynthesis of endogenous IAA. NAA metabolism was also similar to IAA metabolism in the degree to which NAA was conjugated. After only 1 week of treatment, more than 90% of NAA was present as the conjugate. However, NAA did not induce the conjugation of IAA in these experiments, signifying that the ability of the callus to conjugate was specific to each auxin, at least in this experimental system. It has been shown, however, that IAA, NAA, and 2,4-D treatments can promote the conjugation of each other (Südi, 1964, 1966; Johansson, 1972; Venis, 1972), but such experiments included short-term treatments with freshly excised hypocotyl segments as opposed to established callus and organ cultures. The data presented here suggest that in terms of accumulation and conjugation, NAA appears to be intermediate between IAA and 2,4-D. The developmental effects of NAA treatment were also intermediate between those of 2,4-D and IAA (or no auxin treatment). NAA-treated hypocotyls proliferated callus and became organogenic, producing roots, shoots, and embryos.

IAA

In auxin-treated hypocotyls, the most dramatic effect on IAA metabolism was seen in those cultures treated with [$^2H_4$]IAA. After 1 week of treatment, large amounts of total [$^2H_4$]IAA were present in the hypocotyls (Fig. 2). Greater than 99% of [$^2H_4$]IAA was present in conjugated forms at both 1- and 4-week incubation periods. The ability of auxins to induce auxin conjugation has been examined extensively, especially in relation to the formation of IAA-aspartate (Südi, 1966; Johansson, 1972). One working hypothesis is that this pool of conjugated IAA represents a biologically inactive, but not degraded, pool (Cohen and Bandurski, 1982). Experiments involving applications of IAA amino acid conjugates show that these derivatives have IAA activity (Hangarter and Good, 1981; Feung et al., 1977). The auxin activity, however, seems to be due to the presence of the free IAA released from

the hydrolysis of the conjugated form (Hangarter and Good, 1981; Bialek et al., 1983). In addition, recent studies using several plant species have shown that the conjugation of IAA with aspartate can result in the direct catabolism of IAA without prior hydrolysis (Östin, 1995). The very limited callus growth observed in IAA-treated carrot hypocotyls supports the hypothesis that the conjugation of IAA reduces its activity. At the 4-week incubation period, the appearance of the hypocotyls given [$^2H_4$]IAA most closely matched the appearance of the untreated cultures and greatly contrasted with the appearance of both the 2,4-D- and NAA-treated plant material (Fig. 3).

Three conclusions can be drawn from the present experiments. The first conclusion is that exogenous IAA activated the IAA conjugation system, which moderated the internal concentration of the free hormone. The second conclusion concerns the relatively weak effectiveness of IAA in certain long-term experimental systems involving tissue cultures and feeding experiments. Since IAA has been shown to degrade in the presence of both light and nutrient salts (Dunlap and Robacker, 1988), the lack of significant responses to the IAA treatment has been previously attributed to its chemical lability (Gamburg, 1988). However, exogenous free IAA may have reduced biological activity in large part because it is efficiently removed from the free IAA pool by the process of conjugation. The third conclusion is suggested by the concentration of the endogenous IAA after the 4-week incubation with [$^2H_4$]IAA. The concentration of the free and ester conjugates of the endogenous IAA were very low (near the limits of detection), and the pools consisted almost exclusively of the [$^2H_4$]IAA. This result suggests that [$^2H_4$]IAA not only has a positive effect on the promotion of conjugation but also has a negative influence on de novo IAA biosynthesis, which strongly suggests that IAA activates a feedback mechanism for the regulation of its own biosynthesis. As previously suggested, it is also possible that NAA treatment caused a similar inhibition of IAA biosynthesis, as noted by the decreased concentration of IAA in the 4-week NAA-treated hypocotyls.

In summary, these data show that the concentration of IAA was controlled in several different ways in IAA-treated hypocotyls. The free IAA pool was rapidly depleted by the process of conjugation. Over a longer period of time, the presence of exogenous IAA caused apparent feedback inhibition of IAA biosynthesis. Given the high rates of IAA turnover measured in vivo (Tam et al., 1995), inhibited biosynthesis can be expected to decrease IAA pool size. Finally, since the concentration of total IAA was reduced by a factor of 10 from the 1- to 4-week period, it appears that high rates of IAA catabolism also occurred within the hypocotyls. All of these mechanisms would have suppressed the free IAA concentration in IAA-treated hypocotyls, with the result that the effective free IAA pool was not changed enough to induce much callus proliferation, as was seen with the untreated hypocotyls. These effects contrast directly with the abundant callus in the 2,4-D-treated hypocotyls and the considerable callus and organ formation in NAA-treated hypocotyls.

The Role of Auxin in Somatic Embryogenesis

2,4-D and NAA are typically used in different applications because their respective auxin activities are contrasting (Ammirato, 1985). 2,4-D and NAA treatments elicited very different growth patterns in carrot hypocotyls, despite the fact that only minimal changes were measured in the free and conjugated IAA concentrations. Therefore, the developmental effects of treatment with 2,4-D and NAA are most likely due to their ability to act directly as auxins and not due to their ability to perturb IAA metabolism. In previous studies (Michalczuk et al., 1992b, 1992c), we found that 2,4-D-treated hypocotyls and suspension cultures had markedly increased IAA levels compared with controls; however, in the current hypocotyl experiments the changes were more modest.

Transferring callus formed in response to each auxin treatment to fresh medium lacking auxin allowed us to evaluate the embryogenic potential of each callus (Fig. 4). Subsequent to the transfer from 2,4-D-containing medium to fresh medium lacking 2,4-D, the callus started to produce many embryos (Fig. 4A). When NAA-treated hypocotyls were transferred to medium lacking auxins, the callus exhibited a further increase in both embryo and organ formation (Fig. 4B). The low levels of free IAA in control hypocotyls and IAA-treated hypocotyls were insufficient to support the proliferating callus that would be needed as the initiating cells for subsequent embryo development. Thus, although it appears necessary to have a threshold concentration of either endogenous IAA or an exogenous auxin to sustain the initial stage of callus proliferation, the free auxin concentration must fall below a certain maximum to promote the next stage of embryo development. These changes in effective auxin concentration are accomplished by the biochemical reduction of free NAA via conjugation reactions in NAA-treated hypocotyls and the physical removal of the 2,4-D source with the 2,4-D-treated hypocotyls. Indeed, earlier work demonstrated that the 2,4-D concentration is dramatically reduced in developing embryos that are no longer supplied with 2,4-D (Michalczuk et al., 1992b). Finally, we hypothesize that cell culture systems, such as those in certain grasses (Vasil, 1985), which require the reduction but not the elimination of supplied 2,4-D, are likely to have less efficient uptake mechanisms and/or more efficient conjugation processes than carrot cultures. Similar considerations might explain the results of Lou and Kako (1995), who observed that different combinations of exogenous auxins had dramatically different effects on the yield of somatic embryos in cucumber cultures.

The embryogenic potential of the IAA treatment is unclear due to the lack of growth. In the experimental system defined here, there did appear to be a requirement for a synthetic auxin treatment to induce proliferating callus to become competent to produce embryos. It seems reasonable, however, to assume that a high-enough concentration of free IAA by itself should also be able to induce somatic embryogenesis. In fact, some pioneering work in plant tissue culture summarized by Halperin (1995) did demonstrate the ability of IAA to provide long-term carrot cell

cultures from taproot meristems with the potential to form somatic embryos (Levine, 1950). It is conceivable that such cultures exhibited an altered metabolism that would allow IAA to accumulate in a manner similar to the synthetic auxins described here. These experiments underscore the diversity within the experimental system of carrot cell cultures by showing how many different treatments can lead to somatic embryo formation.

NAA and 2,4-D treatments did not appear to affect the biosynthesis of IAA from Trp, based on the incorporation studies using [$^2H_5$]Trp. The ability of synthetic auxins to affect the biosynthesis of IAA from Trp was investigated because our previous study (Michalczuk et al., 1992c) showed a shift in the biosynthetic pathway of IAA from a predominantly Trp-mediated pathway in callus proliferating in the presence of 2,4-D to a predominantly non-Trp-mediated pathway in embryos developing in the absence of 2,4-D. Our present results with [$^2H_5$]Trp show that the Trp pathway continued to remain active in 1-week-old carrot cultures in the presence or absence of auxins. Therefore, the decrease in the Trp pathway for IAA biosynthesis does not depend on a prior 2,4-D treatment, but appears to correlate with the onset of somatic embryo formation.

In conclusion, it seems that the specific experimental system dictates the nature and concentration of the specific endogenous auxins required to promote somatic embryogenesis. The results of this study clearly showed that the exogenous auxins may have profound and unexpected effects on endogenous auxin metabolism. The most striking effect was that IAA application seemed to have a negative effect on the active IAA pool by inducing both IAA conjugation and feedback inhibition of IAA biosynthesis. It seems likely, therefore, that the great diversity of observed auxin effects resides in the metabolic diversity among different experimental systems. Such diversity tends to obscure the identity of the basic requirements for callus proliferation and somatic embryo formation. However, a unifying role of exogenous auxins seems to be illustrated in the carrot system of somatic embryogenesis: an initial high concentration of all free auxins for callus proliferation and a much lower concentration for the organized development of bipolar embryos.

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